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Reversible protein phosphorylation plays a crucial role in the circuitry controlling diverse cellular processes, and the activities of phosphorylating and dephosphorylating enzymes must be carefully balanced in normal cells. It is well documented that many of these enzymes are constitutively expressed, but their activities are tightly regulated by a variety of post translational mechanisms. In the case of the serine/threonine-specific protein phosphatase 2A (PP2A), a catalytic subunit is bound by two regulatory subunits designated A and B. Previously, we established a functional complementation assay for PP2A-C in the yeast *S. cerevisiae* and used this system to isolate two dominant-defective mutants in the human PP2A-Cα gene. We now have generated epitope-tagged forms of the wild-type and mutant alleles and have shown that the epitope-tagged alleles retain biological function in our yeast system. We have also continued the phenotypic analysis of our cell lines expressing PP2A-Cα alleles, using kinase activation assays to assess growth factor responses in these cell lines. These studies will thus increase our understanding of PP2A function in the regulation of proliferation and malignant transformation, and will yield important information about structure-function relationships in the PP2A catalytic and regulatory subunit proteins.

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Alison J. Delong 6/10/99

PI - Signature Date

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INTRODUCTION

Protein phosphatase 2A (PP2A) comprises catalytic (C) and regulatory (A and B) subunits, and a heterotrimeric (ABC) holoenzyme is thought to predominate *in vivo*, although a heterodimeric (AC) form also has been purified (reviewed in Walter and Mumby, 1993). Although the total number of PP2A substrates is not known, it is likely that regulation of PP2A activity is necessary for the correct functioning of signal transduction cascades in which PP2A participates. Essential PP2A subunit functions are strongly conserved throughout evolution, as evidenced by the ability of an *Arabidopsis* PP2A-A subunit gene (*RCN1*) to complement the temperature-sensitive phenotype of a null mutation in the yeast PP2A-A subunit gene (*tpd3*; Garbers et al., 1996). PP2A has not been identified as an oncogene per se, but its interactions with proteins responsible for tumorigenesis are of demonstrated functional significance. Evidence for the role of PP2A in mammalian cell proliferation has originated in studies showing that PP2A is a target for the small-T antigen of SV40 and the small- and medium-T antigens of polyoma virus. These tumor antigens bind the AC heterodimeric PP2A complex (Pallas et al., 1990; Walter et al., 1990) and exclude the B subunit. Interaction with SV40 small t antigen reduces PP2A activity toward the mitogen-activated protein kinases (MAPKs) ERK1 and MEK1, causing deregulation of the MAPK cascade and induction of cell growth (Sontag et al., 1993). Middle T antigen mutants that fail to bind PP2A also are defective in transformation (Campbell et al., 1995). Furthermore, the tumor-promoting agent okadaic acid (OKA; Suganuma et al., 1988) is a potent and specific inhibitor of PP2A and PP1, with PP2A showing approximately 100-fold higher sensitivity (Cohen et al., 1989). The targeting of PP2A by viral T antigens and the tumor-promoting effect of OKA at low concentrations both support the hypothesis that PP2A activity normally suppresses proliferation, and that antagonism of PP2A may be important in tumorigenesis.

Dominant gain-of-function alleles have revealed the identities and functions of many proteins involved in growth control. We proposed to isolate dominant-defective mutants of human PP2A as a tool for investigating potential growth control functions of PP2A in normal and breast cancer cells. Possible causes of dominance for such mutants include competition with the wild-type protein for substrates or for positive regulatory subunits. The PP2A catalytic subunit (PP2A-C) provides a good target for mutagenesis aimed at generation of dominant alleles, because putative catalytic residues have been identified and interactions with positive regulatory subunits are known to be important for normal activity.

Previously, we established a functional complementation assay for PP2A-C in the yeast *S. cerevisiae* and used this system to isolate two dominant-defective mutants in the human PP2A-Cα gene. We had isolated mammalian fibroblastic cell lines expressing mutant and wild-type PP2A-Cα alleles. Our recent efforts have been focused on generating epitope-tagged forms of the mutant alleles and testing those alleles for biological function in our yeast system. We have performed preliminary microscopic characterization of yeast cells expressing mutant and wild-type PP2A-Cα alleles. We have also continued the phenotypic analysis of our cell lines expressing PP2A-Cα alleles, using kinase activation assays to assess growth factor responses in these cell lines.

BODY

Construction of epitope-tagged human PP2A-C subunits in S. cerevisiae

We have previously shown that *S. cerevisiae* can be used as a rapid in vivo assay system for human PP2A-C activity. Mutation of a conserved histidine residue in the PP2A-Cα sequence

(the Cα-H118N mutant) confers a strong dominant-defective phenotype; mutation of a conserved arginine residue (the Cα-R89A mutant) results in a weaker dominant-defective phenotype. A detailed description of this work was recently accepted for publication in *Gene*; a copy of the manuscript is appended.

Several alternative hypotheses can be invoked to explain the dominance of the H118N mutant in yeast cells. First the mutant enzyme might bind substrate efficiently and prevent the endogenous yeast PP2A-C subunit from interacting with one or more essential substrate(s). Second, the mutant enzyme might interact normally with PP2A regulatory subunits (A and/or B) that are required for normal activity of the yeast PP2A-C subunit. Third, the mutant enzyme might bind another protein (neither substrate nor A or B regulatory subunit) not bound by the wild-type enzyme. Fourth, expression of the heterologous PP2A-C might down-regulate the endogenous PP2A-C expression; in the presence of the catalytically inactive H118N mutant, this would be lethal. However, we find that the levels of endogenous yeast PP2A-C are stable as transgene PP2A-Cα accumulates when mutant or wild-type PP2A-Cα expression is induced with galactose and PP2A-C accumulation over a time course is monitored by immunoblotting,. Thus the last hypothesis can be eliminated. We are currently using immunoprecipitation techniques to investigate whether mutant and wild-type PP2A-Cα bind PP2A-A and -B regulatory subunits in yeast.

We have initiated microscopic characterization of yeast cells expressing wild-type and dominant defective PP2A-Cα alleles. Our preliminary analysis of cells expressing the H118N allele reveals abnormal bud morphologies similar to those exhibited by PP2A-depleted cells (Ronne et al., 1991) or *cdc55* mutant cells (Healy et al., 1991), consistent with the hypothesis that these cells undergo growth arrest due to loss of PP2A function. We have not observed abnormal phenotypes in cells expressing the wild-type PP2A-Cα allele.

Our earlier experiments, as well as published reports (Baharians and Schonthal, 1998), indicated that it may be difficult to isolate mammalian cell lines expressing high levels of wild-type PP2A-C. To facilitate analysis of expression levels in mammalian cells we decided to construct epitope-tagged PP2A-C alleles. PCR amplification was used to add an amino-terminal FLAG epitope tag to wild-type and H118N PP2A-Cα coding sequences, producing the FLAG-PP2A-C fusion sequence shown below. To ascertain whether the epitope tag altered the

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G G A T T C C C G G G C C A C C ATG GAC TAC AAG GAC GAC GAT GAC AAT GGC GGC ATG GAC GAG AAG..
  (BamHI)              (Kozak)   M   D   Y   K   D   D   D   D   K   G   G   M   D   E   K...
```

biological activity of our PP2A-Cα constructs, we introduced these alleles into our galactose-inducible yeast expression vectors and assayed their activity in yeast cells. Wild-type FLAG-Cα supports growth of our temperature-sensitive yeast strain, indicating that the FLAG-tagged C subunit provides complementing phosphatase activity. FLAG-Cα-H118N exhibits a dominant-defective phenotype comparable to that of the native Cα-H118N parent. Immunoblot analysis shows that the FLAG-PP2A-C proteins are detectable both with anti-PP2A-C antibody, and with anti-FLAG antibody. The FLAG-tagged protein appears to be stable in yeast cells; we see no evidence of proteolytic cleavage of the FLAG tag to release 'native' PP2A-Cα protein. Thus the FLAG epitope-tagged PP2A-Cα constructs appear to maintain their biological activity in yeast cells, and are suitable for our studies in mammalian cells. We are currently generating a set of FLAG-Cα constructs for use in mammalian cells.

Expression of wild type and dominant-defective PP2A -C alleles in mammalian cells
We have used our stably transfected fibroblast cell lines to assess effects of expressing PP2A-Cα+ and PP2A-Cα-H118N in the well-characterized normal fibroblastic cell line TGR1 (Prouty

et al., 1993). We chose the Cα-H118N mutant because of its stronger dominant-defective phenotype. We have used two Cα+ cell lines that show two- to four-fold elevation over basal PP2A-C levels, and four Cα-H118N cells lines that show seven- to 25-fold elevation over basal PP2A-C levels. We have assayed mitogen-activated protein kinase (MAPK) activation by immunoblotting with phospho-specific antibodies to gauge responses to serum stimulation. We see no consistent alterations in serum responses in our PP2A-Cα-expressing cell lines, when compared to cell lines carrying an empty vector construct. Our data suggest that expression of PP2A-C alleles in these cell lines do not alter MAPK cascade activity in response to serum. It is likely that the high growth factor concentrations found in serum would preclude detection of any subtle changes in MAPK activation, and a more sensitive assay would employ specific growth factors at concentrations that stimulate MAPK activation to half-maximal levels. However, our results must be interpreted carefully in light of recently published data suggesting that PP2A-C expression levels are tightly regulated in mammalian cells (Baharians and Schonthal, 1998). Such a regulatory mechanism may contribute to the difficulty of isolating cell lines expressing high levels of wild-type transgene PP2A-Cα. Furthermore, okadaic acid treatment was shown to cause increased PP2A-C accumulation. Thus it is possible that expression of a dominant-interfering Cα-H118N allele also causes increased expression of the endogenous PP2A-C, and the elevated PP2A-C levels we have measured in our Cα-H118N cell lines may include both transgene-encoded C subunits and additional endogenous C subunits. The difficulty of quantitating transgene expression levels in these cell lines was one of the major reasons for creating epitope-tagged constructs. We are also focusing on strategies to allow isolation of cell lines expressing increased PP2A-Cα+ and PP2A-Cα-H118N levels. We will re-examine the question of MAPK activation in our new cell lines using an improved growth factor response assay.

In the course of our serum stimulation experiments, we observed abnormal cell cycle profiles in fluorescence-activated cell sorting (FACS) analysis of the PP2A-Cα+ cell line which showed the greater elevation of PP2A-C level (three- to four-fold) in immunoblots. FACS profiles for this cell line suggested either an accumulation of tetraploid cells or an accumulation of cells in the G2 and/or M phases of the cell cycle. Cell cycle profiles for our other cell lines were generally normal. While no significant conclusions can be drawn from preliminary analysis of the phenotype of a single cell line, we will be careful to screen our new PP2A-Cα+ cell lines for similar phenotypes.

KEY RESEARCH ACCOMPLISHMENTS

- Further characterization of dominant-defective alleles of human PP2A-C
- Development of functional epitope-tagged PP2A-C alleles
- Assays of MAPK activation in response to serum stimulation in cell lines expressing wild-type and dominant-defective PP2A-C alleles

REPORTABLE OUTCOMES

- Lizotte, D., D. D. McManus, H. R. Cohen, and A. DeLong. 1999 Functional expression of human and *Arabidopsis* protein phosphatase 2A in *Saccharomyces cerevisiae* and isolation of dominant-defective mutants. *in press (Gene)*
- "Studying PP2A function in yeast and mammalian cells" Donna Lizotte, Kimberly

Hemond, David McManus and Alison DeLong; poster presentation at the Brown University MCB Graduate Program Annual Retreat, Sept. 2, 1998.

- "Using dominant defective mutants to study protein phosphatase 2A function in vivo" Molecular Genetics and Development seminar presented by Alison DeLong to the Biology Department, McGill University, Montreal, Quebec, Canada.

- "Characterizing a dominant-defective mutant of human protein phosphatase 2A" undergraduate honors thesis prepared by Kimberly Hemond, Brown University Division of Biology and Medicine.

- Development of stable cell lines expressing wild-type and dominant-defective PP2A-C alleles.

CONCLUSIONS

We have shown that *S. cerevisiae* can be used as a rapid in vivo assay system for human PP2A-C activity. Mutation of a conserved histidine residue in the PP2A-Cα sequence confers a strong dominant-defective phenotype. Addition of an amino-terminal FLAG epitope tag does not interfere with the biological activity of the human PP2A-Cα subunit in yeast. Our data indicate that expression wild-type and dominant-defective mutant PP2A-C subunits in mammalian cells do not grossly perturb MAPK activation in response to serum stimulation, however, accurate quantitation of transgene PP2A-C expression levels in these cells is not practicable. MAPK activation will be further investigated in cell lines expressing epitope-tagged PP2A-C alleles.

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Functional expression of human and *Arabidopsis* protein phosphatase 2A in *Saccharomyces cerevisiae* and isolation of dominant-defective mutants

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Key Words: functional complementation / catalytic subunit / mutational analysis / dominant-negative

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Abbreviations: AthC1, AthC3 and AthC5, *Arabidopsis thaliana* PP2A-C genes; *CDC55*: *S. cerevisiae* PP2A B regulatory subunit gene; Clb2-Cdc28, B-type cyclin/cyclin-dependent kinase; cs, cold sensitive; HsCα and HsCβ, human PP2A-C genes; kDA, kilodaltons; PCR, polymerase chain reaction; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2A-C, PP2A catalytic subunit; PP2B, protein phosphatase 2B; *PPA2*, *S. pombe* PP2A-C gene; *PPH21* and *PPH22*, *S. cerevisiae* PP2A-C genes; *PPH3*, *S. cerevisiae* PP2A-like phosphatase gene; *RTS1*, *S. cerevisiae* PP2A B' regulatory subunit gene; SDS-PAGE, SDS polyacrylamide gel electrophoresis; *TPD3*, *S. cerevisiae* PP2A A regulatory subunit gene; ts, temperature sensitive; YPD, yeast peptone glucose medium.

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ABSTRACT

Protein phosphatase 2A (PP2A), a heterotrimeric serine/threonine-specific protein phosphatase, comprises a catalytic subunit and two distinct regulatory subunits, A and B. The primary sequence of the catalytic (C) subunit is highly conserved in evolution, and its function has been shown to be essential in yeast, *Drosophila* and mice. In many eukaryotes, the C subunit is encoded by at least two nearly identical genes, impeding conventional loss-of-function genetic analysis. We report here the development of a functional complementation assay in *S. cerevisiae* that has allowed us to isolate dominant-defective alleles of human and *Arabidopsis* C subunit genes. Wild-type human and *Arabidopsis* C subunit genes can complement the lethal phenotype of *S. cerevisiae* PP2A-C mutations. Site-directed mutagenesis was used to create two distinct, catalytically impaired C subunit mutants of the human and *Arabidopsis* genes. In both cases, expression of the mutant subunit in yeast prevented growth, even in the presence of functional C subunit proteins. This dominant growth defect is consistent with a dominant-interfering mode of action. Thus we have shown that *S. cerevisiae* provides a rapid system for the functional analysis of heterologous PP2A genes, and that two mutations that abrogate phosphatase activity exhibit dominant-defective phenotypes in *S. cerevisiae*.

1. INTRODUCTION

Protein phosphatase 2A (PP2A) is a member of the PPP family of protein serine/threonine phosphatases (Barford, 1996). Purification of PP2A enzymatic activity from a wide variety of species typically results in the isolation of the PP2A catalytic (C) subunit in a complex with one or more regulatory subunits. A heterotrimeric holoenzyme comprising regulatory A and B subunits bound to the catalytic subunit is thought to predominate in vivo. Interactions between the catalytic and regulatory subunits have been shown to modulate PP2A activity both in vivo and in vitro (reviewed in Mumby and Walter, 1993). Recent evidence shows that other proteins can bind to the holoenzyme, or in some cases replace one or both regulatory subunits, resulting in altered PP2A activity (see, for example, Murata et al., 1997). The catalytic subunit exhibits strong sequence similarity to protein phosphatase 1 (PP1) in regions that constitute the putative active site (Goldberg et al., 1995). Although the reaction mechanisms of PP1 and PP2A have not yet been defined, amino acid sequence conservation, crystallographic analysis of the PP1 active site (Goldberg et al., 1995) and biochemical analysis of the bacteriophage λ phosphatase (Zhuo et al., 1994) have identified residues required for metal ion coordination, substrate binding, and catalysis (see Fig. 1).

The amino acid sequences of C and A subunits are highly conserved in eukaryotes as distantly related as *Arabidopsis thaliana*, humans, and yeast. Conservation of A subunit function has been demonstrated previously by complementing a *S. cerevisiae* A subunit mutant with an *Arabidopsis* A subunit gene (Garbers et al., 1996). In the animals studied to date, both the C and A subunits are expressed ubiquitously and appear to be encoded by small and quite homogeneous gene families. In plants, however, A and C subunit gene families are larger; the *Arabidopsis* genome encodes at least 5 C subunits and 3 A subunits (reviewed in Smith and Walker, 1996; Stamey and Rundle, 1996). In both plants and animals, different B subunit isoforms are encoded by two or more unrelated gene families, some of which are expressed in a tissue-specific manner. Thus the more variable B subunit may dictate substrate specificity (Mayer-Jaekel and Hemmings, 1994), an hypothesis supported by data showing that the tumor antigens of DNA tumor viruses replace B subunits in PP2A complexes and alter the enzyme's catalytic properties (Yang et al., 1991; Cayla et al., 1993; Sontag et al., 1993).

PP2A activity has been shown to play a negative role in growth-control in systems such as *Xenopus, S. pombe*, and *Drosophila* (Lee et al., 1991; Kinoshita et al., 1993; Mayer-Jaekel et al., 1994). In *S. cerevisiae*, however, PP2A is required for entry into mitosis (Lin and Arndt, 1995). Two PP2A-C genes, designated *PPH21* and *PPH22* have been identified in *S. cerevisiae*. Inactivation of both genes is detrimental but not lethal unless a gene encoding a PP2A-like phosphatase, *PPH3*, is also mutated (Sneddon et al., 1990; Ronne et al., 1991; Lin and Arndt, 1995). Cells carrying a temperature-sensitive (ts) *pph21-102* allele in a *pph21 pph22 pph3* null background retain viability but exhibit G2 cell cycle arrest, reduced B-type cyclin/cyclin-dependent (Clb2-Cdc28) kinase activity and abnormal bud morphologies at the non-permissive temperature (Lin and Arndt, 1995). The PP2A-A subunit is encoded by a single gene, *TPD3*, and *tpd3* null mutants exhibit a Ts^- phenotype (van Zyl et al., 1992). Two B subunits have been identified; mutants in the 55kD B subunit gene *CDC55* are cold-sensitive (cs), while mutants in the 56 kD B' subunit gene *RTS1* are both ts and cs as well as ethanol-hypersensitive (Healy et al., 1991; Shu et al., 1997). It is unclear why the C subunit is essential while the A and B regulatory subunits are only conditionally required.

The fact that each PP2A subunit is encoded by a gene family in many eukaryotes presents a significant impediment to conventional genetic analysis, and suggested to us that the isolation of dominant PP2A mutants might provide an alternative to the isolation of mutations in individual gene family members followed by the generation of multiply mutant stocks. Taking advantage of the high sequence conservation exhibited by PP2A genes and the availability of PP2A mutant strains of yeast, we have developed a functional assay for the isolation and characterization of dominant-defective alleles of PP2A catalytic subunit genes. We have demonstrated the utility of this system by the construction of dominant-defective PP2A-C mutants from *Arabidopsis thaliana* and from humans.

2. MATERIALS AND METHODS

2.1 Yeast strains and plasmid constructs

Yeast strains used in this work are listed in Table 1. Yeast growth media and genetic techniques were as described by Ausubel et al. (1992). After lithium acetate transformation, yeast transformants were

selected at 25° on synthetic complete glucose (SCD) medium lacking tryptophan and uracil. Segregants that had lost either the *pph21-102*/YCp50 construct or the heterologous C subunit construct were isolated by growing cells non-selectively (on YPD or YPGalactose medium at 25°) followed by replica-patching single colonies onto selective (SCD or SCGalactose) medium with or without uracil or tryptophan. Segregants were colony-purified and their phenotypes verified by restreaking on the appropriate media.

All *GAL* constructs used in this work are derivatives of either YCp22GAL or YEp112GAL (shown in Table 1 as *pGAL/TRP/CEN* and *pGAL/TRP/2μ*, respectively), isogenic yeast expression vectors carrying the *GAL1/GAL10* bidirectional promoter, a *TRP1* selectable marker, and a *CEN* or 2μ circle origin of replication, respectively (Pitluk et al., 1995). Plasmid pADL302 (*GAL10:AthC1/TRP/2μ*) was constructed by subcloning the AthC1 coding sequence from pPP2A-1 (kind gift of Sabine Rundle) into the *Eco*RI site of YEp112GAL. Plasmids pDMC2 and pDMC16 (*GAL1:HsCα/TRP/CEN* and *GAL1:HsCα/TRP/2μ*) were constructed as follows: the HsCα coding sequence was amplified from plasmid pUC.HPP2ACαRM1 (kind gift of N. Andjelkovic and B. Hemmings) and given *BamH*I cloning ends via polymerase chain reaction (PCR), followed by cleavage with *BamH*I and ligation into *Bam*HI-cleaved YCp22GAL and YEp112GAL, respectively. The primers used were 5' GCGGATCCTTACAGGAAGTAGTCTGGGG 3' and 5' CGGGATCCATGGACGAGAAGGTGTTCACCAAGG 3'. Similarly, plasmid pDMC4 (*GAL1:HsCβ/TRP/CEN*) was constructed by PCR amplification of the HsCβ coding sequence from plasmid pTZ18U.HFP6A2Cβ (kind gift of N. Andjelkovic and B. Hemmings), followed by *BamH*I subcloning into YCp22GAL, using PCR primers 5' GCGGATCCTTATAGGAAGTAGTCTGGGG 3' and 5' CGGGATCCATGGACGACAAGGCGTTCACCAAGG 3'.

To make the ADH:AthC1 constructs, a 2 kb *Bam*HI fragment carrying the ADH promoter and terminator was subcloned from pAAH5 (Ammerer, 1983) into pTZ19 (US Biochemical). The AthC1 coding sequence was ligated into the unique *Hin*dIII site between the ADH promoter and terminator. To make plasmids pADL316 (*ADH:AthC1/TRP/CEN*) and pADL317 (*ADH:AthC1 antisense/TRP/CEN*), sense and anti-sense ADH:AthC1 fusions were subcloned as *Sph*I fragments into

YCplac22 (Gietz and Sugino, 1988). To chromosomally integrate the ADH:AthC1 construct, the pADL316 *Sph*I fragment was cloned into the integrating plasmid YIplac128 (Gietz and Sugino, 1988), followed by addition of a G418 resistance cassette (*Sma*I-*Sac*I fragment) from pFA6-KanMX4 (Wach et al., 1994). The resulting plasmid (pADL340) was linearized with *Afl*II and transformed into strain CY3007, with selection for G418 resistance.

2.2 Site-directed mutagenesis

Mutants were generated using an oligonucleotide-mediated mutagenesis protocol (Deng and Nickoloff, 1992). The following mutagenic oligonucleotide primers were used. oAthC1-H115N: 5' CTGACGGCTCTCATTATTCCCTCGCAGG 3'; oAthC1-R86A: 5' CAGAATAGTAGCCAGCATCTACGTAATCTCCCATGAAG 3'; oHsCα-H118N: 5' TTCTTCGAGGGAATAATGAGAGCAGACAG 3'; oHsCα-R89A: 5' GGGAGATTATGTTGATGCAGGATATTATTCAGTT 3'.

2.3 Immunoblot analysis

Standard SDS-PAGE and immunoblotting protocols were followed (Ausubel et al., 1992). The polyclonal anti-PP2A-C antibody used was raised against a conserved carboxy-terminal peptide (KVRRTPDYFL), and was the kind gift of T. Stukenberg and M. Kirschner. Peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) was detected using a standard chemiluminescence protocol.

3. RESULTS

3.1 *Arabidopsis* and Human PP2A-C clones complement a yeast PP2A mutant

We established a functional complementation assay for PP2A activity in *S. cerevisiae*. Strain CY3007 carries null alleles in both PP2A-C genes and in the *PPH3* gene, which encodes a related phosphatase; lethality of this triple mutation is rescued by a low copy-number plasmid bearing a temperature sensitive (ts) *pph21* allele (*pph21-102*; Lin and Arndt, 1995). CY3007 cells grow at 25° or 30°, but not at 35°. We cloned *Arabidopsis* and human cDNAs encoding PP2A-C subunits into yeast

expression vectors and tested their ability to complement the temperature sensitivity of strain CY3007. When expressed from the constitutive alcohol dehydrogenase (ADH) promoter and carried on a low copy-number plasmid, the *Arabidopsis* AthC1 cDNA (see Fig. 1; (Ariño et al., 1993) complemented the Ts^- phenotype of CY3007, indicating that the *Arabidopsis* PP2A-C subunit is functional in yeast (Fig. 2). When cloned in the antisense orientation, the AthC1 cDNA failed to complement in CY3007 (Fig. 2).

To exclude the possibility that the yeast ts allele contributed to complementation, we grew cells carrying the ADH-AthC1 plasmid non-selectively and screened for tryptophan or uracil auxotrophy to identify segregants that had lost either plasmid. Loss of the ADH-AthC1 plasmid conferred a Trp^- phenotype and restored the parental Ts^- phenotype. Loss of the *pph21-102* plasmid conferred a Ura^- phenotype, but the resulting cells still grew at 35°, as well as 25° and 30° (Fig. 2). Thus the ADH-AthC1 plasmid is necessary and sufficient for complementing the Ts^- phenotype, and is capable of supporting normal growth in the absence of any yeast PP2A-C protein.

Expression of either the AthC1 or the human HsCα cDNA (see Fig. 1; Stone et al., 1988) under control of a galactose-inducible *GAL* promoter also complemented the Ts^- phenotype of CY3007. Cells expressing either the *Arabidopsis* or human cDNA grew at 25° on both glucose- and galactose-containing media and at 35° on galactose, but did not grow on glucose at 35° (Fig. 3A and B). The sufficiency of the *GAL:*AthC1 construct was tested by segregating either the *GAL:*AthC1 or YCp50 *pph21-102* plasmid. Again, loss of the plasmid carrying the *Arabidopsis* C1 gene restored the parental Ts^- phenotype. Loss of the *pph21-102* plasmid conferred a galactose-dependent phenotype; segregants were viable at 25°, 30° and 35° only on galactose-containing media (data not shown). Surprisingly, the human HsCβ gene (see Fig. 1; Hemmings et al., 1988) did not provide complementing PP2A activity in yeast (Fig. 3B). Failure to complement was not due to poor expression of the HsCβ protein, since similar amounts of the HsCα and β subunit proteins were detected in galactose-grown cells (Fig. 4A). Thus expression of either the AthC1 or HsCα (but not HsCβ) catalytic subunit gene provides PP2A function in yeast cells.

We predict that cells expressing a catalytically active C subunit that was unable to interact with the endogenous yeast regulatory subunits (e.g. *TPD3* or *RTS1*) would exhibit phenotypes characteristic of loss-of-function mutations in those genes. Both *tpd3* and *rts1* mutations prevent growth at 37°C (van Zyl et al., 1992; Shu et al., 1997), therefore we tested the ability of cells expressing human and *Arabidopsis* PP2A-C subunits to grow 37°. Cells carrying a *GAL:*HsCα or *GAL:*AthC1 construct were viable at 37°C, suggesting that these heterologous C subunits functionally interact with the yeast A and B' regulatory subunits (Fig. 3C). Cells carrying the *GAL:*AthC1 construct formed small colonies at this temperature. This small colony phenotype may indicate that the AthC1 protein has lower affinity for Tpd3p than does HsCα, however, it is also possible that the catalytic activity of AthC1 is reduced at 37°C, a temperature above the normal growth range of *Arabidopsis.*

High-level over-expression of PP2A-C mRNA has been shown to be toxic in *S. cerevisiae*, but cells expressing the *PPH22* gene under control of the *GAL1* promoter grow at a normal rate (Ronne et al., 1991). We have not observed strong negative growth effects of *GAL*-driven expression of wild-type human or *Arabidopsis* PP2A-C genes. In fact, under permissive conditions cells carrying a high copy number *GAL:*C construct grow slightly faster in liquid media (ca. 2.5 hr doubling time) than do cells carrying the vector alone (ca. 3 hr doubling time).

3.2 Isolation of dominant defective alleles of PP2A-C

We hypothesized that mutation of an amino acid residue involved in catalysis would produce a dominant-defective PP2A-C allele if substrate binding and/or subunit interactions were unaffected by the lesion. We chose a putative active site histidine (H115 in the *Arabidopsis* gene and H118 in the human sequence; see Fig. 1) and mutated this residue to asparagine using site-directed mutagenesis (see section 2.2). Mutations at the analogous position abrogate catalytic activity but not substrate binding in calcineurin (PP2B) and λ phosphatase (Zhuo et al., 1994; Mertz et al., 1997), two enzymes which exhibit close similarity to PP2A in putative active site sequences. To test the activity of the mutant proteins, we expressed the mutant alleles under control of *GAL* promoters in strain CY3007, and assayed their effects on growth (Fig. 5). Cells carrying the *GAL:*AthC1-H115N construct were unable to grow on either galactose or glucose at 35° (Fig. 5A), indicating that the mutant enzyme is

defective and provides no catalytic activity at 35°. More importantly, cells carrying this construct were unable to grow on galactose at 25°, the permissive temperature for the *pph21-102* allele (Fig. 5A) or at 30° (data not shown). Thus the defective phenotype of the H115N allele is dominant over the ts yeast PP2A allele and prevents growth at all temperatures tested. We have obtained the same results with the H118N mutant of the human Cα subunit (Fig. 6B and data not shown).

To test the dominance of the H115N allele with respect to the homologous wild-type gene, we chromosomally integrated a wild-type AthC1 gene under control of the ADH promoter. The resulting strain, DLY6, grew well at 30° or 35° on glucose- or galactose-containing media (Fig. 5B), indicating that the integrated ADH-AthC1 fusion supplies PP2A activity. DLY6 cells carrying the *GAL:*AthC1-H115N construct on a high copy-number plasmid grew on glucose-containing media at either 30° or 35°, but failed to grow on galactose at either temperature (Fig. 5B). Thus the H115N allele is dominant over a wild-type AthC1 allele. The expression level of the mutant protein influenced the strength of the dominant-defective phenotype, as a low copy-number AthC1-H115N plasmid caused a weaker, but still clearly detectable inhibition of growth (Fig. 5C). This copy number effect is consistent with the dominant-interfering activity of the mutant proteins.

To show that both the mutant and endogenous PP2A-C subunits are expressed in galactose-grown cells, we used immunoblotting to assay PP2A-C subunit levels after galactose induction of cells carrying mutant and wild-type *GAL10:*AthC1 constructs (Fig. 4B). We have obtained the same results with wild-type and H118N mutant human HsCα (data not shown). Wild-type and mutant PP2A-C proteins accumulate to comparable levels after galactose induction, and appear equally stable over a nine-hour time course (data not shown).

To determine whether other active site mutations also would confer a dominant-defective phenotype, we introduced a lesion at a position thought to be essential for binding of the phosphorylated substrate. Conversion of an active site arginine to alanine in the λ phosphatase causes a 20-fold decrease in binding of a synthetic substrate and a 500-fold catalytic defect (Zhuo et al., 1994). We introduced the same mutation (R86A in the *Arabidopsis* protein and R89A in the human protein; see

Fig. 1) into our wild-type *GAL:*PP2A-C constructs and tested the activity of the mutant constructs as described above. Cells carrying AthC1-R86A failed to grow on either glucose or galactose at 35° (Fig. 6 and data not shown), showing that, like the H115N allele, this mutant provides no complementing phosphatase activity at 35°. Expression of the R86A allele also blocked growth of CY3007 cells at 25° (Fig. 6A). The R86A mutant protein accumulates to levels comparable to those of the wild-type and H118N proteins after galactose induction (data not shown). These data show that the R86A mutation is also dominant-defective, and we have obtained similar results with the human HsCα-R89A mutant (data not shown). In contrast, the HsCβ provides no complementing phosphatase activity at 35°, but does not exhibit a dominant-defective phenotype (Fig. 6B).

4. DISCUSSION

We have used yeast to screen for dominant-defective mutants of the PP2A catalytic subunit. We have shown that both *Arabidopsis* and human PP2A genes complement a ts mutation in a yeast PP2A-C gene. Constitutive or inducible expression of a heterologous PP2A-C subunit gene is sufficient to allow growth of yeast cells in the absence of any yeast PP2A-C protein. Site-directed mutagenesis of the *Arabidopsis* and human PP2A-C genes yielded catalytically inactive alleles that exhibited dominant phenotypes. Expression of the dominant-defective phenotype was influenced by copy number, consistent with a dominant-interfering effect of the mutant gene products.

The robust growth of yeast cells in which PP2A function is supplied by a heterologous C subunit protein indicates that sequences required for specific and functional interaction with essential substrates are conserved between the yeast, *Arabidopsis* and human PP2A-C subunits. The predicted amino acid sequences of the C subunit gene products show a very high degree of similarity, 80-90% over the length of the *Arabidopsis* and human sequences. Both yeast proteins carry amino-terminal extensions that are approximately 70 amino acids in length and serve unknown functions. Our work shows that the extensions are not required for essential PP2A functions in *S. cerevisiae*, since the *Arabidopsis* and human clones are expressed from their native start codons. Complementation also reveals that critical recognition features of the substrate proteins are conserved. The full range of

PP2A substrates required for growth of yeast cells is not known, but the available evidence suggests that essential substrates include proteins involved in bud morphogenesis, cytoskeletal organization and entry into mitosis (Ronne et al., 1991; Lin and Arndt, 1995; Evans and Stark, 1997). Finally, we tested the competence of the human and *Arabidopsis* C subunits to interact with the endogenous yeast regulatory subunits by assaying for growth at the restrictive temperature for the *tpd3* (A subunit) and *rts1* (B' subunit) mutants. Our data support the hypothesis that sequences required for interaction with regulatory subunits also are conserved in these heterologous proteins, consistent with earlier observations that an *Arabidopsis* A regulatory subunit complements a *tpd3* null allele in cells expressing wild-type yeast C subunits (Garbers et al., 1996).

Although the human HsCα subunit supports growth of yeast, the HsCβ subunit does not provide complementing PP2A activity in *S. cerevisiae*. The non-complementing phenotype of the Cβ subunit construct is surprising because only eight residues differ in the predicted amino acid sequences of the Cα and Cβ subunits. Most of the substitutions are conservative changes in sequences not known to be essential for PP2A function. However, it has recently been shown that mice homozygous for a PP2A-Cα null mutation die during embryogenesis, despite significant levels of Cβ subunit expression (Götz et al., 1998). Thus failure of the Cβ subunit to complement in yeast may reflect functional specificity in vivo.

We used site-directed mutagenesis to create two dominant-defective alleles of PP2A (H115N or H118N and R86A or R89A). Target residues for mutagenesis were chosen on the basis of putative active site function (Goldberg et al., 1995) and biochemical analysis of mutants of the bacteriophage λ phosphatase (Zhuo et al., 1994). Both mutants were inactive in our complementation assay, indicating loss of catalytic activity. The H115N/H118N mutant exhibited a strong dominant-defective phenotype, while the R86A/R89A mutant displayed a slightly weaker dominant-defective phenotype. Preliminary microscopic characterization of cells expressing the H115N/H118N allele reveals abnormal bud morphologies similar to those exhibited by PP2A-depleted cells (Ronne et al., 1991) or *cdc55* mutant cells (Healy et al., 1991), consistent with the hypothesis that these cells undergo growth arrest due to loss of PP2A function (D. Lizotte and A.

DeLong, unpublished). The H115N mutant of AthC1 was dominant in the presence of a constitutively expressed wild-type copy of AthC1, showing that the dominant-defective phenotype was not specific to the Pph21-102p protein. A partially dominant mutant of *S. pombe* PP2A-C has been isolated previously. A high dose of this cold-sensitive (cs) *ppa2* allele was shown to arrest growth under non-permissive conditions, and to retard growth and reduce cell size under permissive conditions (Kinoshita et al., 1990). The basis of the mutant's partial dominance is unknown. However, expression of the cs allele increased okadaic acid sensitivity mildly, suggesting a dominant-interfering mechanism (Kinoshita et al., 1993).

The severity of the dominant growth defect phenotype was clearly affected by the copy number of the mutant and wild-type genes, consistent with the hypothesis that the dominant-defective enzyme competes with the functional C subunits for substrates or regulatory subunits. Either a substrate sequestration or a regulatory subunit titration model could account for the data we have presented here. The HsCα-H118N mutant has been independently constructed and shown to be catalytically inactive despite normal substrate binding characteristics (Heekyoung Chung and David Brautigan, personal communication). Equivalent mutants of λ phosphatase and calcineurin display the same biochemical characteristics, but have not been assayed for phenotypes in vivo (Zhuo et al., 1994; Mertz et al., 1997). In contrast, a λ phosphatase mutant equivalent to the R86A/R89A mutant constructed here exhibits a 20-fold decrease in substrate binding and a catalytic defect less severe than that of the histidine mutant (Zhuo et al., 1994). The weaker phenotype of the R86A/R89A mutant may indicate that substrate binding contributes to, but is not essential for the dominant phenotype. Biochemical analysis will be required to resolve these questions. Interestingly, the HsCβ subunit does not provide complementing PP2A activity, but is not dominant-defective. This result suggests that the factors required for functional complementation also are required for the dominant-defective phenotype, and argues against the hypothesis that any non-functional C subunit may exert a dominant effect. The partially dominant mutation conferring cold sensitivity on *S. pombe* PP2A-C maps to a conserved region, but does not affect a residue known to play a role in substrate binding or catalysis.

An alternative hypothesis is that the expression of heterologous PP2A-C subunit proteins down-regulates synthesis of endogenous C subunit protein. Loss of Pph21p expression would be tolerated in the presence of functional heterologous C subunits, but would be lethal in the presence of catalytically inactive mutants. A post-transcriptional autoregulatory mechanism limiting PP2A-C expression in mammalian cells has been reported recently (Baharians and Schonthal, 1998), and it is unclear whether a similar mechanism is operative in yeast. However, we have not observed down-regulation of endogenous C subunit protein levels in cultures expressing wild-type, dominant-defective, or non-complementing C subunits under *GAL* promoter control (D. Lizotte and A. DeLong, unpublished). Furthermore, a significant level of PP2A-C over-expression has been achieved in *S. pombe* (Kinoshita et al., 1993), and induction of high levels of PP2A-C mRNA is lethal in *S. cerevisiae*, suggesting that the C subunit protein is indeed over-expressed (Ronne et al., 1991). Thus down-regulation of endogenous C subunit levels does not account for the dominant-defective phenotype.

We have shown that yeast provides a functional assay system for heterologous PP2A-C genes; this system is likely to be generalizable, given the high conservation of C subunit sequences. We have isolated dominant-defective mutants of PP2A genes from *Arabidopsis* and humans, and we plan to use these mutants to analyze PP2A functions in whole plants and in mammalian tissue culture cells. This genetic system may also prove useful for analyzing interactions between heterologous or homologous catalytic and regulatory subunits of PP2A.

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TABLES

Table 1. Strain list

W303	*MATa ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	K. Arndt
CY3007	*MATa pph22::HIS3 pph21::HIS3 pph3::LEU2 [pph21-102/URA/CEN] W303*	Lin and Arndt (1995)
DLY6	*MATa pph22::HIS3 pph21::HIS3 pph3::LEU2 LEU2::ADH:AthC1 kan^r [pph21-102/URA/CEN]*	this work
DLY8	*CY3007 [ADH:AthC1/TRP/CEN]*	this work
DLY9	*CY3007 [ADH:AthC1-antisense/TRP/CEN]*	this work
DLY10	*CY3007 [pGAL/TRP/CEN]*	this work
DLY11	*CY3007 [pGAL/TRP/2μ]*	this work
DLY12	*CY3007 [GAL10:AthC1/TRP/2μ]*	this work
DLY13	*CY3007 [GAL10:AthC1-H115N/TRP/2μ]*	this work
DLY14	*CY3007 [GAL10:AthC1-R86A/TRP/2μ]*	this work
DLY16	*CY3007 [GAL1:HsCα/TRP/2μ]*	this work
DLY17	*CY3007 [GAL1:HsCα/TRP/CEN]*	this work
DLY18	*CY3007 [GAL1:HsCβ/TRP/CEN]*	this work
DLY19	*CY3007 [GAL1:HsCα-H118N/TRP/CEN]*	this work
DLY20	*CY3007 [GAL1:HsCα-R89A/TRP/CEN]*	this work
DLY21	*DLY6 [pGAL/TRP/2μ]*	this work
DLY22	*DLY6 [GAL10:AthC1/TRP/2μ]*	this work
DLY23	*DLY6 [GAL10:AthC1-H115N/TRP/2μ]*	this work
DLY24	*DLY6 [pGAL/TRP/CEN]*	this work
DLY25	*DLY6 [GAL10:AthC1/TRP/CEN]*	this work
DLY26	*DLY6 [GAL10:AthC1-H115N/TRP/CEN]*	this work

FIGURE LEGENDS

Figure 1. Comparison of PP2A catalytic subunit amino acid sequences

Residues likely to be critical active site residues are underlined (Goldberg et al., 1995). The substitutions found in catalytic subunit mutants are shown below the consensus sequence, with bold letters indicating mutations generated in this work. Also shown are *pph21* and *pph22* mutations

conferring temperature sensitivity in *S. cerevisiae* (†; Lin and Arndt, 1995; Evans and Stark, 1997) and a *ppa2* mutation conferring cold sensitivity in *S. pombe* (*; Kinoshita et al., 1990). The sites of several mutations conferring okadaic acid resistance also are indicated (^; Shima et al., 1994; Kaneko et al., 1995). Numbering above each sequence block refers to the amino acid sequence of Pph22p, while italicized numbering below the sequence blocks refers to the sequence of the human gene products. Two additional *Arabidopsis* catalytic subunit sequences (AthC3 and AthC5) were included in the sequence comparison, but are not shown in the figure. Residues conserved in six of the seven sequences compared are shown in the consensus sequence (CONSEN). Sequence identities and conservative substitutions are represented by upper case letters, while non-conservative substitutions are shown in lower case. The sequence of the peptide used to raise the anti-PP2A-C antibody used in these studies is shown in italics under the carboxy-terminal end of the consensus sequence. GenBank accession numbers PPh21p: X56261; Pph22p: X56262; AthC1: M69732; AthC3: M96841; AthC5: U39568; HsCα: M36951; HsCβ: X12656. The alignment and consensus sequence were generated using the PILEUP and PRETTY algorithms of the University of Wisconsin GCG package.

Figure 2. Growth of strains carrying ADH-AthC1 plasmids

Yeast cells from single colonies were patched on duplicate plates containing YPD medium and grown at the temperatures shown. The PP2A-C alleles carried by cells in each set of patches are shown (top): Ts+, parental strain W303; ts, temperature-sensitive mutant strain CY3007; ts + C1, DLY8; ts + anti-C1, DLY9; (ts+ C1) - ts, Ura- derivatives of DLY8; (ts + C1) - C1, Trp- derivatives of DLY8. Similar results were obtained in streak-out tests of growth of these strains.

Figure 3. Growth of yeast expressing heterologous C subunits under *GAL* promoter control

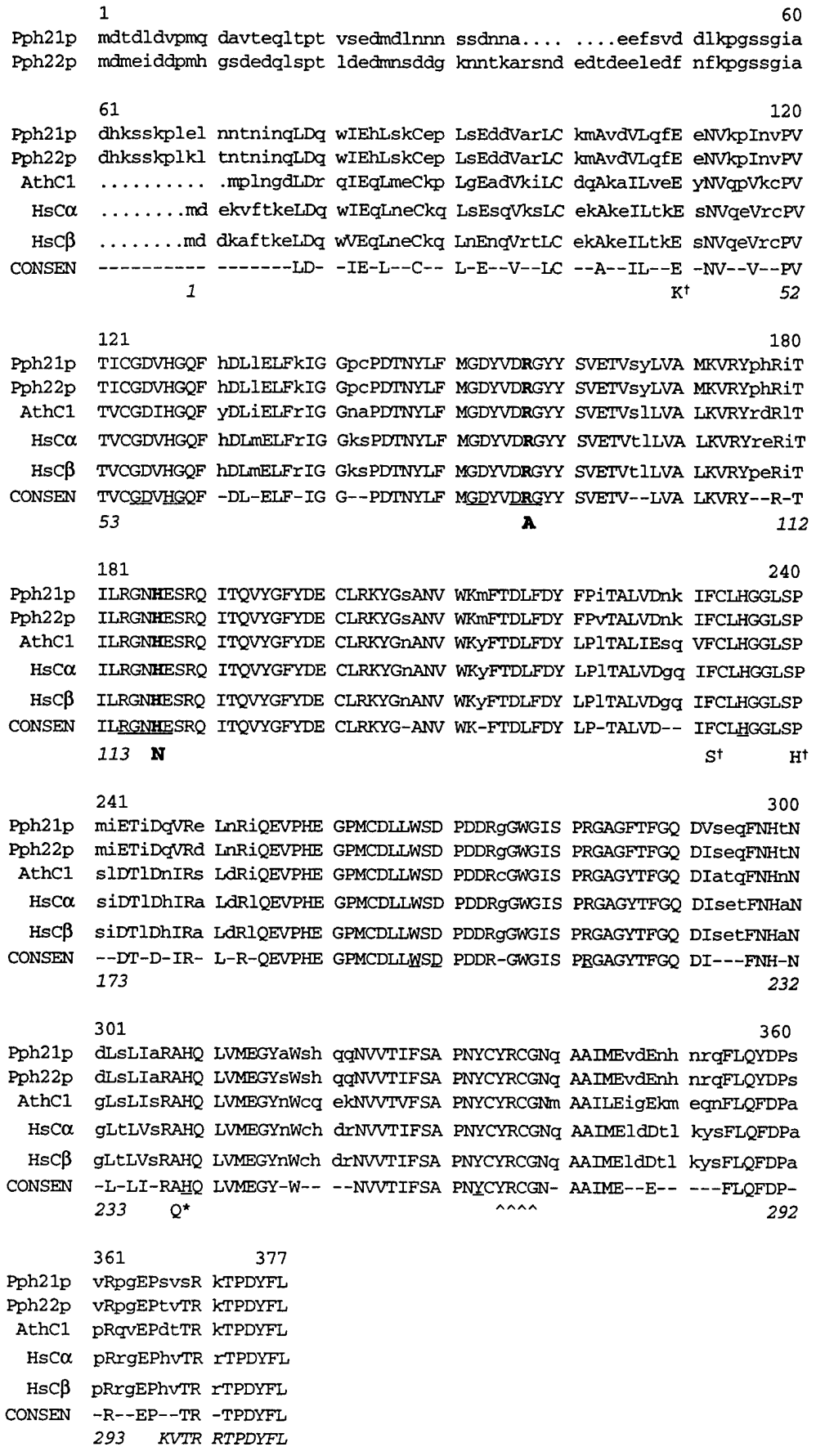
CY3007 transformants carrying a *GAL10:*AthC1 construct (AthC1; DLY12) or an empty vector (-; DLY11) were streaked on duplicate plates containing selective glucose and galactose medium and incubated at the temperature indicated (A). CY3007 transformants carrying an empty vector (sector 1; DLY11) or low copy (sector 2; DLY17) or high copy (sector 4; DLY16) *GAL1:*HsCα, or low copy *GAL1:*HsCβ (sector 3; DLY18) were streaked on duplicate plates containing selective glucose and galactose medium and incubated at the temperature indicated (B). CY3007 transformants carrying

an empty vector (quadrant 5; DLY11), low copy (quadrant 6; DLY17) or high copy (quadrant 7; DLY16) *GAL1:*HsCα, or high-copy *GAL10:*AthC1 (quadrant 8; DLY12) were streaked on selective galactose medium and incubated at the temperature indicated (C).

Figure 4. *GAL*-driven expression of wild-type and dominant mutant C subunits in yeast
Cells carrying an empty vector (-; DLY10), and low copy *GAL1:*HsCα (DLY17) and *GAL1:*HsCβ (DLY18) constructs were grown in galactose, harvested, lysed, and total soluble proteins were extracted and subjected to immunoblot analysis (A). Wild-type and mutant AthC1 proteins were detected after induction of the *GAL10* promoter (B). Cells carrying an empty vector (-; DLY11), *GAL10:*AthC1 (DLY12) or *GAL10:*AthC1-H115N (DLY13) were grown in selective raffinose medium, diluted into selective galactose medium at time 0, and samples were harvested after 1 and 3 hours, as indicated. Cells were lysed and proteins were extracted and analyzed as described above. The positions of the yeast C subunit (Pph21p) and the human (HsC) and *Arabidopsis* (AthC) C subunit proteins are indicated on the right of each panel.

Figure 5. Growth of yeast cells carrying AthC1-H115N constructs
CY3007 (A) and DLY6 (B and C) transformants carrying an empty vector (-), a *GAL10:*AthC1 construct (+) or a *GAL10:*AthC1-H115N construct (H115N) were streaked on duplicate plates containing selective glucose and galactose medium and incubated at the temperature indicated. Plasmid carried high-copy number (2μ circle; A and B) or low-copy number (centromeric; C) origins of replication. Strains used: DLY11, DLY12, DLY13 (A); DLY21, DLY22, DLY23 (B); DLY24, DLY25, DLY26 (C). Similar results were obtained in streak-out tests of growth of these strains.

Figure 6. Growth of yeast cells carrying dominant PP2A-C mutants
CY3007 transformants carrying wild-type (+), and mutant (H115N and R86A) *GAL10:*AthC1 constructs (panel A; strains DLY12, DLY13 and DLY14) or wild-type and mutant (H118N) *GAL1:*HsCα constructs or wild-type *GAL1:*HsCβ (panel B; strains DLY17, DLY19 and DLY18) were streaked on duplicate plates containing selective galactose medium and incubated at the temperature indicated. Similar results were obtained in streak-out tests of growth of these strains.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CONSEN ---------- -------LD- -IE-L--C-- L-E--V--LC --A--IL--E -NV--V--PV
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121 180
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181 240
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241 300
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233 Q* ^^^^ 292
361 377
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AthC1 pRqvEPdtTR kTPDYFL
HsCα pRrgEPhvTR rTPDYFL
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Figure 1

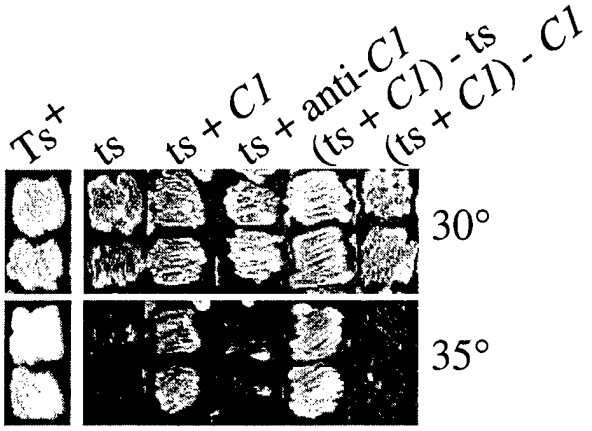
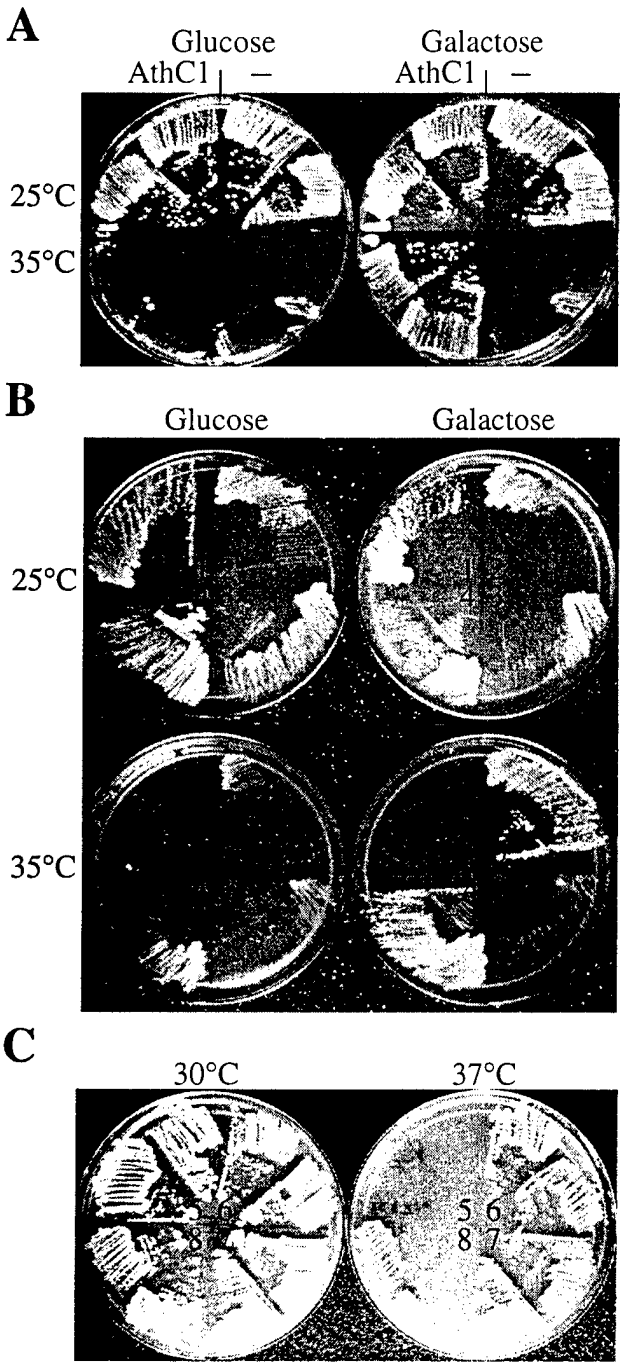


Figure 2



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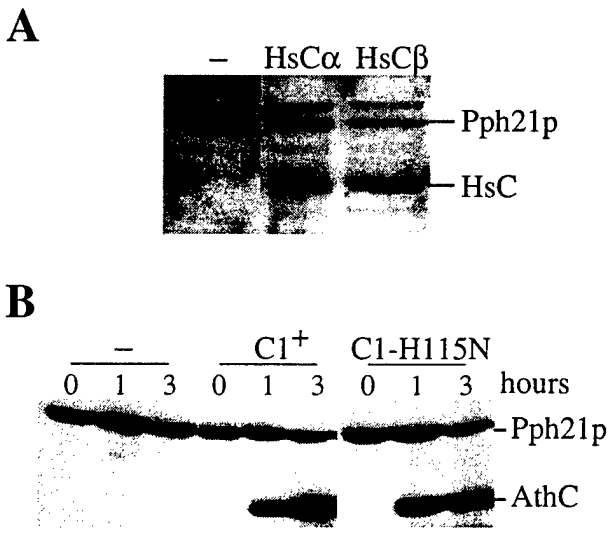

Figure 4

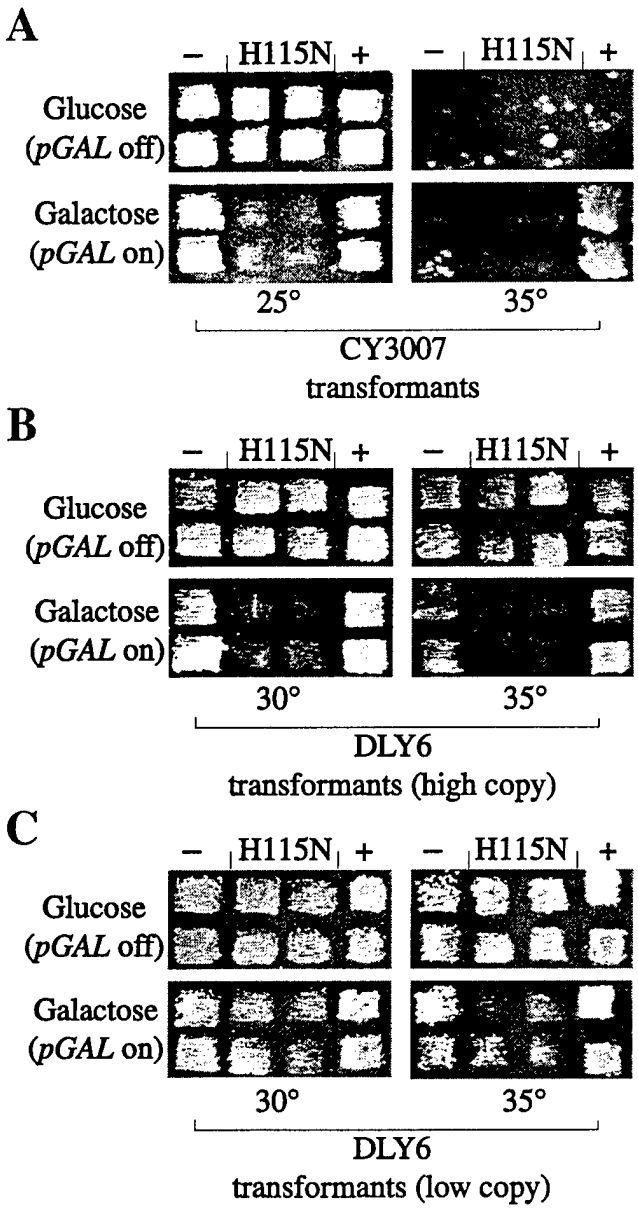

Figure 5

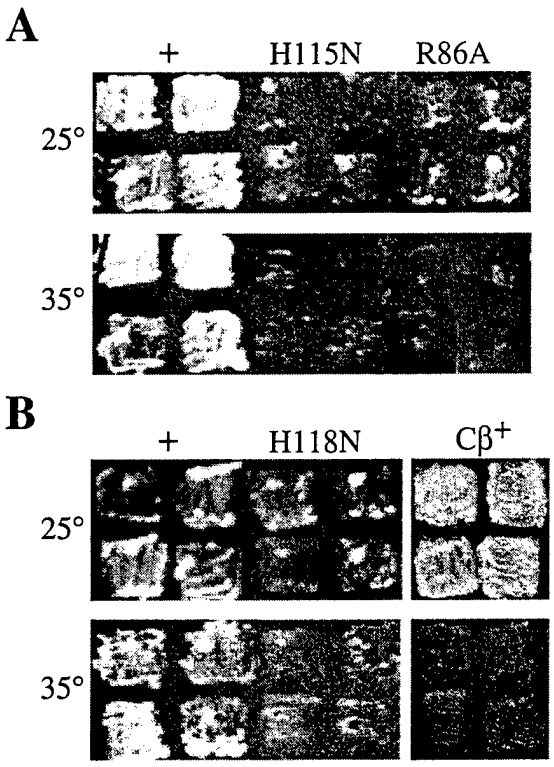

Figure 6